

IN THE DRAWINGS

Please replace Figure 1A with substitute Figure 1A submitted herewith.

N.B.  
I cannot  
sub Figs.

REMARKS

Applicants have amended the description and claims to correct inadvertent typographical errors. At page 30, line 33, "JNK3a1" has been replaced with "JNK3 $\alpha$ 1"; at page 31, line 4, "JNK 1" has been replaced with "JNK1", and at line 18, "JNK3a1" has been replaced with "JNK3 $\alpha$ 1"; at page 33, line 28, "CuKa" has been replaced with "CuK $\alpha$ "; and at page 45, line 27, "sequence" has been replaced with "sequence". In addition, since applicants will not be filing color drawings, the phrases describing color in the specification at page 44, lines 7, 9, 11, 12, 22, 23, 26, 29-33 and page 45, lines 5-7, 11, 22, 25 have been deleted. Applicants have submitted herewith copies of original pages 30, 31, 33, 44 and 45 with the corresponding handwritten amendments. Applicants have also amended claim 11, step (b) to replace "machin-reable" with "machine-readable". Support for this amendment can be found in claim 11 as originally filed.

Applicants have also amended Figure 1A to correct an inadvertent typographical error. The amendment replaces the amino acid sequence "ILRGLKYIHSAD" with "ILRGLKYIHSAN" at the beginning of the third line of the ERK2 sequence.

This amendment is supported by the reference to ERK2 (T.G. Boulton et al., Cell, 65, pp. 663-675 (1991), submitted herewith as Exhibit A) at page 43, lines 19-20 of the specification as originally filed. At page 664 of that reference, the correct amino acid sequence is shown as "IleLeuArgGlyLeuLysTyrIleHisSerAlaAsn" at residues 3-14 of line 6 in Figure 1(A). Applicants have also submitted original Figure 1A with the corresponding handwritten amendment.

None of these amendments adds new matter.

#### Conclusion

Applicants request entry of the amendments and early allowance of the claims.



Respectfully submitted,



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## APPENDIX

### CLAIMS

11. A computer for determining at least a portion of the structure coordinates corresponding to X-ray diffraction data obtained from a molecule or molecular complex, wherein said computer comprises:

a) a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein said data comprises at least a portion of the structural coordinates of the JNK3 complex according to Figure 1;

b) a machine-readable data storage medium comprising a data storage material encoded with [machin-reable] machine-readable data, wherein said data comprises X-ray diffraction data obtained from said molecule or molecular complex;

c) a working memory for storing instructions for processing said machine-readable data of (a) and (b);

d) a central-processing unit coupled to said working memory and to said machine-readable data storage medium of (a) and (b) for performing a Fourier transform of the machine readable data of (a) and for processing said machine readable data of (b) into structure coordinates; and

e) a display coupled to said central-processing unit for displaying said structure coordinates of said molecule or molecular complex.

entities. This approach enables the determination of the optimal sites for interaction between chemical entities, including candidate JNK3 inhibitors and JNK3. For example, high resolution X-ray diffraction data collected from crystals exposed to different types of solvent allows the determination of where each type of solvent molecule resides. Small molecules that bind tightly to those sites can then be designed and synthesized and tested for their JNK3 inhibition activity.

All of the complexes referred to above may be studied using well-known X-ray diffraction techniques and may be refined versus 1.5-3 Å resolution X-ray data to an R value of about 0.20 or less using computer software, such as X-PLOR [Yale University, ©1992, distributed by Molecular Simulations, Inc.; see, e.g., Blundell & Johnson, supra; Meth. Enzymol., vol. 114 & 115, H. W. Wyckoff et al., eds., Academic Press (1985)]. This information may thus be used to optimize known JNK3 inhibitors, and more importantly, to design new JNK3 inhibitors.

In order that this invention be more fully understood, the following examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

#### EXAMPLE 1

##### Expression and purification of JNK3

A BLAST search of the EST database using the published JNK3 $\alpha$ 1 cDNA [S. Gupta et al. (1996)] as a query identified an EST clone (#632588) that contained the entire coding sequence for human JNK3 $\alpha$ 1. Polymerase chain reactions (PCR) using pfu polymerase (Stratagene) were used to introduce restriction sites into the cDNA for cloning into the pET-15B expression vector at the NcoI and BamHI sites for expression of the protein in *E.*

*coli*. Due to the poor solubility of the expressed full length protein (Met 1-Gln 422), an N-terminally truncated protein starting at Ser residue at position 40 (Ser 40), corresponding to Ser 2 of ~~JNK1~~<sup>JNK1</sup> and JNK2 proteins [S.

5 Gupta et al. (1996)], preceded by Met (initiation) and Gly residues, was produced. The Gly residue was added in order to introduce an NcoI site for cloning into the expression vector. Further, systematic C-terminal truncations were performed by PCR to identify a construct that give rise to diffraction-quality crystals. This  
10 construct, which was prepared by PCR using deoxyoligonucleotides 5'

GCTCTAGAGCTCCATCGGCAGCAAAAGCAAAGTTGACAA 3' (forward primer with initiation codon underlined) and 5'

15 TAGCGGATCCTCATTCTGAA TTCATTACTTCCTTGTA 3' (reverse primer with stop codon underlined) as primers and confirmed by DNA sequencing, encodes amino acid residues Ser40-Glu402 of JNK3 <sup>$\alpha$</sup> ~~1~~, preceded by Met and Gly residues, was used for structural studies described in this paper. Control  
20 experiments indicated that the truncated JNK3 protein has an equivalent kinase activity towards myelin basic protein when activated with an upstream kinase MKK7 in vitro (unpublished results).

*E. coli* strain BL21 (DE3) (Novagen) transformed  
25 with the JNK3 expression construct was grown at 30°C in shaker flasks into log phase (OD600 ~ 0.8) in LB supplemented with 100 µg/ml carbenicillin. IPTG was then added to a final concentration of 0.8 mM and the cells were harvested 2 hours later by centrifugation.

30 *E. coli* cell paste containing the truncated JNK3 protein was resuspended in 10 volumes/g lysis buffer [50 mM HEPES, pH 7.2, 10% glycerol (v/v), 100 mM NaCl, 2 mM dithiothreitol (DTT), 0.1mM PMSF, 2 µg/ml Pepstatin, 1µg/ml each of E-64 and Leupeptin]. Cells were lysed on  
35 ice using a microfluidizer and centrifuged at 100,000 x g for 30 min at 4°C. The 100,000 x g supernatant was

activity when activated by MKK7 *in vitro*. All crystallographic studies were carried out using this form of the enzyme.

Crystallization trials were performed by combining the hanging-drop vapor diffusion technique and a sparse matrix search, in the presence and absence of MgAMP-PNP. No crystals were obtained in the absence of MgAMP-PNP, while crystallization trials carried out in the presence of MgAMP-PNP yielded an orthorhombic crystal form at 20°C over a reservoir solution containing 18-20% (v/v) polyethylene glycol monomethyl ether (average  $M_r$  = 550), 10% (v/v) ethylene glycol, 20mM  $\beta$ -Mercaptoethanol and 100mM Hepes (pH 7.0). The crystallization droplet contained a mixture of 1 $\mu$ L of reservoir solution plus 1mL of a protein solution that had been preincubated for one hour with 1mM AMP-PNP and 2mM MgCl<sub>2</sub> on ice. The crystals belong to the orthorhombic space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> ( $a$ =51.50 Å,  $b$ =71.24 Å and  $c$ =107.60 Å) with one enzyme molecule per asymmetric unit. The solvent content of the crystal is 44%. Before data collection, crystals were equilibrated in their reservoir solution for 2-5 minutes before flash-frozen in nitrogen gas for X-ray data collection at -170°C.

### Example 3

#### X-Ray data collection and structure determination

X-ray data were measured on an Raxis IIC image plate, with mirror-focused CuK $\alpha$  X-rays generated by a rotating anode source. The diffraction images were processed with the program DENZO and data scaled using SCALPACK [Z. Otwinowski, In "Data Collection and Processing", L. Sawyer, N. Isaacs and S.W. Bailey, eds., Warrington, U.K.: Science and Engineering Council/Daresbury Laboratory. pp. 55-62 (1993)]. The data processing statistics are summarized in Table 1.

for  $\beta\beta$  strands. Disordered regions are indicated with dashed lines. Both JNK3 and cAPK sequence numbering are shown. Phosphorylation sites in the phosphorylation lip are denoted by an asterisk. JNK3 residues that differ from JNK1 and JNK2 are highlighted in bold.

Fig 2a is a ribbon representation of the overall fold of JNK3 complexed with MgAMP-PNP. ~~Blue~~ The structure indicates secondary structural elements and loops conserved among protein kinases, <sup>as well as</sup> ~~Magenta indicates~~ extensions and insertion characteristic of MAP kinases. The JNK insertion and the phosphorylation lip are ~~colored cyan and red, respectively.~~ <sup>shown</sup> The disordered region (residues 212-216) is indicated with dotted lines. Bound AMP-PNP and two  $Mg^{2+}$  ions are represented by space-filling models. The C $\alpha$  positions of the regulatory phosphorylation sites Thr221 and Tyr223 are shown and labeled. Secondary structural elements are labeled according to ref. This diagram was constructed using RIBBONS [M. Carson, J. Appl. Cryst., 24, pp. 958-61 (1987)].

Figure 2b is a stereoscopic view of the superimposed structures of JNK3/MgAMP-PNP and Erk2 C $\alpha$  representations of the structures of JNK3 ~~(yellow and red)~~ and Erk2 ~~(blue and white)~~ are shown after superposition of their C-terminal domains. Segments with largest structural divergence are labeled and highlighted ~~in red and white, respectively.~~

Fig 3 is stereoscopic view of the superimposed structures of JNK3 and cAPK. C $\alpha$  representation of JNK3 ~~(yellow and red)~~ and cAPK ternary complex ~~(blue, white and green)~~ are shown after superposition of their C-terminal domains. The phosphorylation lip ~~is colored red~~ in JNK3 and ~~white~~ in cAPK, and the PKI inhibitor ~~is colored red,~~ showing the difference in the conformation of the lip between two enzymes, and the lip of JNK3 occupying part of the peptide binding channel. MnAMP-PNP in cAPK ternary complex is omitted from the drawing and only the kinase catalytic core portion of cAPK is shown.

Figure 4a is stereoscopic view of the active site of JNK3. Molecules of AMP-PNP and  $Mg^{2+}$  are shown together with their surrounding JNK3 residues. The AMP-PNP molecule is shown as thick bonds, and the protein residues as thin bonds. Two  $Mg^{2+}$  ions (~~colored orange and~~ labeled M1 and M2) and two water molecules (~~colored cyan and~~ labeled W1 and W2) are shown as spheres. Hydrogen bonds are indicated by dashed lines.

Fig 4b is a detailed comparison of the active site of JNK3 with that of cAPK.  $\alpha$  representation of the ATP binding sites of JNK3 (~~yellow~~) and cAPK (~~blue~~) with the side chains of selected residues included. The atoms of AMP-PNP in the JNK3 binary complex and cAPK ternary complex have been superimposed. The N-terminal domains of the two enzymes are well aligned, while the difference in domain orientation results in the misalignment of the catalytic residues clustered in C loop and DFG loop, such as Asp189 and Asp207, with those in the N-terminal domain, such as Lys93.

Fig 5 is a substrate binding specificity of JNK isoforms. The solvent accessible surface of JNK3 is shown with the PKI inhibitor (drawn as ~~orange~~ tube) after the same superposition of JNK3 and cAPK structures done in Fig 3. Surface area corresponding to the JNK3 residues not conserved in JNK1 and JNK2 are ~~colored red~~ <sup>shaded</sup>. Two clusters of divergent regions of JNK isoforms identified from the amino acid ~~sequence~~ <sup>sequence</sup> alignment are located next to each other on the protein surface in the C-terminal lobe. The area containing  $\alpha$ F and L13 has been shown to direct the substrate binding specificity toward cJun.